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Comm.
Dr. Bing
Dr. Cattell
Dr. Loosli

THE COUNCIL FOR TOBACCO RESEARCH – U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

Date: 1/23/73

1. Principal Investigator (give title and degrees):

Elliot S. Vesell, A.B., M.D., Professor and Chairman, Dept. Pharmacology, and Professor of Medicine and Genetics
G. Thomas Passananti, B.S., M.S., Ph.D., Asst. Professor, Dept. Pharmacology

2. Institution & address:

Pennsylvania State University College of Medicine
Milton S. Hershey Medical Center
Hershey, Pennsylvania 17033

3. Department(s) where research will be done or collaboration provided:

Pharmacology

4. Short title of study:

Radioimmunoassay for Nicotine

5. Proposed starting date: June 1, 1973

6. Estimated time to complete: One year

7. Brief description of specific research aims:

As mentioned in my application to the Council for Tobacco Research dated July 1, 1970, the objectives of this study are to "determine the rate of elimination of nicotine from the blood and urine of smokers and nonsmokers, to define the range of individual variation of nicotine metabolism in these two groups and to determine whether chronic nicotine administration causes induction of the hepatic microsomal drug-metabolizing enzymes responsible for biotransformation of nicotine." To achieve these aims, plasma levels of nicotine will have to be determined after nicotine is administered in various forms. We plan to measure rates of nicotine decay from plasma (nicotine pharmacokinetics). We are concerned with the effects of different types of environment (previous exposure to nicotine, particularly) on nicotine kinetics. These kinetic data on nicotine decay from plasma would be obtained in naive and confirmed cigarette smokers, cigar smokers and pipe smokers. Thus variations in nicotine kinetics would be examined not only within each group but also among the different groups. Hopefully, this would provide data not previously available on the role of different types of environment on rates of nicotine decay from plasma.

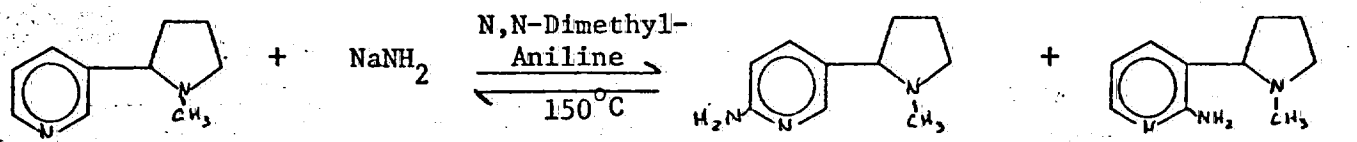
Such kinetic studies on nicotine metabolism have not previously been performed but short time points of blood sampling ranging from 2 minutes to 30 minutes after exposure to nicotine would be obtained initially because we anticipate a very rapid decline in nicotine blood concentrations after nicotine is absorbed. Nicotine decay from blood would be investigated in certain of these studies after the individuals inhaled several puffs of a cigarette or after 15 minutes of pipe or cigar smoking. To perform this study a rapid, extremely sensitive nicotine assay is required.

8. Brief statement of working hypothesis:

Large individual differences may exist in the rates at which smokers metabolize nicotine and also in the rates at which nonsmokers metabolize nicotine. We plan to investigate rates of nicotine metabolism in smokers and nonsmokers to determine whether such large individual variations exist in each group. If they do exist, the heavy smoker who is a slow metabolizer of nicotine might be exposed to higher and more prolonged blood and tissue levels of the drug than the rapid nicotine metabolizer. This possible difference in capacity to metabolize nicotine might render the slow nicotine metabolizer more liable to the pharmacological effects of nicotine. To investigate pharmacokinetic differences among individuals in nicotine metabolism, a radioimmunoassay for nicotine is being developed.

9. Details of experimental design and procedures (append extra pages as necessary)

After a brief unsatisfactory experience with 6-OH nicotine (which we found unsuitable to conjugate with albumin), we decided to synthesize another nicotine derivative that could be directly conjugated to albumin. The 6-amino-nicotine was synthesized according to the method of Chichibabin (Chem. Abstracts 19:69M, 1925) with several modifications in the scheme of purification:



nicotine + sodium amide = 6-amino nicotine + 2-amino nicotine

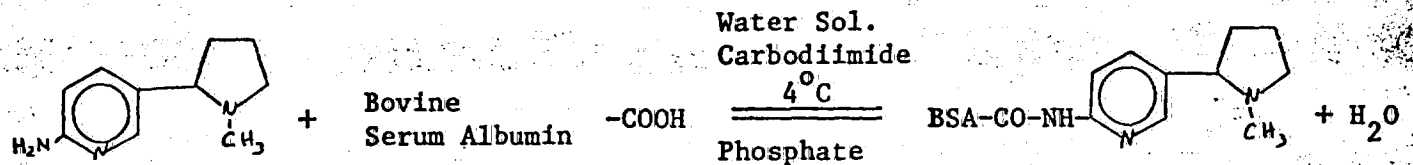
The progress of the reaction was monitored with thin layer chromatography (TLC), using Methylene Chloride : Methanol, 4:1 as the solvent system and both silica gel and aluminum oxide as coatings for the plates. The spots were visualized either with short wave U.V. light or with Iodine vapor. Purification by column chromatography was attempted as separation on the plates was good. However, there was little correlation between separation on TLC and on column chromatography, probably because decomposition took place on the column even though the columns were protected from direct light.

Rapid column chromatography was substituted and proved satisfactory, provided the extracted reaction mixture was purified partially by Kugelruhr distillation prior to chromatography. Two isomers of the amino nicotine, shown in the above equation, were found as predicted by Chichibabin. However, since his original paper was published in 1925 we decided to verify the assignment of structure of the two compounds by both infrared and NMR spectroscopy. The purity of the compounds was proved by these techniques and further assured by elemental analysis: the actual percentages agreed exactly with theoretically expected values. The 2-amino isomer was easily isolated, but the 6-amino isomer was considerably more difficult to isolate in crystalline form due to lack of the intra molecular hydrogen bonding present in the 2-amino compound. In addition, the 6-amino nicotine isomer is especially sensitive to light or air oxidation and is hygroscopic. It was partially purified by Kugelruhr distillation followed by rapid column chromatography, recrystallization from isopropyl ether and finally sublimation.

Several attempts were made to conjugate 6-amino-nicotine directly to bovine serum albumin (BSA) by a carbodiimide condensation procedure similar to that used by Spector and Parker (Science 168:1347, 1970), for their conjugation of morphine to BSA. The conjugate was examined by U.V. spectroscopy following ultrafiltration. The mole ratio of nicotine to BSA was not determined.

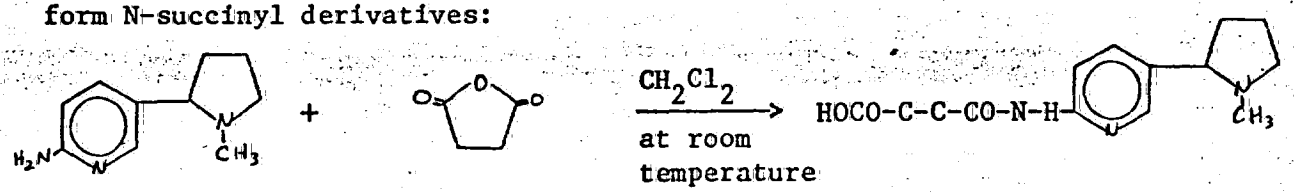
9. Details of experimental design and procedures (cont'd.)

All immunizations were made in rabbits in suspensions of complete Freund's Adjuvant. Booster injections in multiple sites followed at the third week after injection. Samples were withdrawn weekly beginning two weeks after the booster injection. The antiserum was screened for nicotine sensitive antibodies with the Ouchterlony immunoprecipitation technique. The results were negative in all cases. Failure was thought to be due to too close approximation of the nicotine molecule to the albumin to permit recognition of the nicotine hapten by the antibody forming mechanism:



6-amino nicotine

A new approach was therefore selected to place the nicotine hapten further away from the albumin molecule and thereby facilitate its recognition by the antibody forming process. Succinic anhydride reacts with amino groups to form N-succinyl derivatives:



6-amino nicotine Succinic anydride N-succinyl-6-amino-nicotine

This is a relatively long chain with no molecular substitutions so that it should have the smallest antigenic effects of its own. This compound has been synthesized and conjugation achieved:

N-succinyl-6-amino-nicotine + BSA*(NH_2) ⟶ protein-NH-CO-C-C-CO-NH-nicotine

N-succinyl-6-amino-nicotine-protein conjugate

*also done with porcine and bovine thyroglobulin

The conjugate was injected into rabbits one week ago with the expectation that an antibody to nicotine will be formed. Crossreactivity of such an antibody to cotinine will be tested. Although crossreactivity is expected, the extent to which cotinine and nicotine coexist in human blood for short periods after cigarette or cigar smoking can be determined independently by TLC. At very short periods after inhalation of smoke, cotinine will probably not be present in significant concentrations since more time would be required for its production by metabolism of nicotine. Thus, use of the radioimmunoassay would hopefully detect nicotine without its metabolites over short time periods after smoke inhalation. Fortunately for this project, nicotine disappears very rapidly from blood so a pharmacokinetic study of nicotine seems feasible to conduct by means of a radioimmunoassay for nicotine, even though crossreactivity of the antibody with cotinine may occur.

Following initial characterization, rabbit antinicotine sera will be further examined for titre, sensitivity and affinity, according to the method of Berson and Yallow (Clin. Chim. Acta 22:51, 1968). A standard curve will then be prepared from each antiserum; percent of radioactive nicotine bound to antibody is plotted against varying known concentrations of nicotine added in the presence of a constant predetermined amount of labelled nicotine. Cross reactance of such

9. Details of experimental design and procedures (cont'd.)

chemically related compounds as cotinine, nicotinamide, and nicotinic acid will be determined for each antiserum by preparing standard curves for each compound.

The development of the nicotine radioimmunoassay at this stage should be relatively straightforward and will proceed along the following lines:

Selection of appropriate volumes of antisera, sample sera and tritiated nicotine will be determined. The necessity of maintaining sufficient radioactivity levels for efficient counting over the physiological nicotine concentration range will influence choice of these volumes.

Optimum times and conditions for incubation of antisera, sample sera, and tritiated nicotine vary with individual antisera and must be worked out empirically.

Separation of the bound and free phases is accomplished by adsorption of the free phase to either silica gel, dextran coated charcoal or similar adsorbent.

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Laboratories of the Department of Pharmacology comprise 9000 square feet, including a cold room. Major equipment available therein includes:
Gilford Model 2400-S with recorder
Beckman Model DBG
Beckman Model DU2
Sorvall Model RC2B Centrifuge
Two Beckman L265B Centrifuges
International Model CL Centrifuge
Beckman DPM-100 Liquid Scintillation Counter
Aminco-Bowman Spectrofluorometer
Beckman Zeromatic SS-3 pH Meter
Dubnoff-Metabolic Shaking Incubator
Several Mettler P1200 Balances
Buchiflashevaporator
Brinkman Thin-Layer Equipment
High Temperature Oven
High-Speed Liquid Chromatograph, duPont 830
Glowall 320 Gas Chromatograph with 3 different detector systems

11. Additional facilities required:

None

12. Biographical sketches of investigator(s) and other professional personnel (append):

Appended

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

Appended

14. First year budget:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
Elliot S. Vesell	10	---
G. Thomas Passananti	100	21,000
Technical		
Caroll Haines	50	3,000
	Sub-Total for A	24,000
B. Consumable supplies (by major categories)		
Reagents (including $500 for tritiated nicotine)		2,000
Rabbits for antibody		600
Glassware		600
	Sub-Total for B	3,200
C. Other expenses (itemize)		
Human volunteers (30 @ $100)		3,000
	Sub-Total for C	3,000
	Running Total of A + B + C	30,200
D. Permanent equipment (itemize)		
	Sub-Total for D	
	E	4,530
E. Indirect costs (15% of A+B+C)	Total request	34,730

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2						
Year 3						

16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

None

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

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Signature Elliot S Vesell MD Date 1/23/73

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Responsible officer of institution

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Title Assistant Provost for Grants and Contracts
Hershey Medical Center

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